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Potential role of Nanos3 in maintaining the undifferentiated spermatogonia population

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Abstract

Nanos gene encodes for zinc-finger protein with putative RNA-binding activity which shows an evolutionary conserved function in germ cell development. In the mouse, three Nanos homologs have been identified: Nanos1, Nanos2 and Nanos3. The Nanos3 ortholog is expressed in both male and female gonads of early embryo and, after birth, it is found only in the testis. Nanos3 targeted disruption results in the complete loss of germ cells in both sexes; however the role of Nanos3 in the testis during the postnatal period has not been explored yet.

In this study, we show that, in prepuberal testis, Nanos3 is expressed in undifferentiated spermatogonia and that its up-regulation causes accumulation of cells in the G1 phase, indicating that this protein is able to delay the cell cycle progression of spermatogonial cells. This is in line with the observation that the cell cycle length of the undifferentiated germ cells is longer than in differentiating spermatogonia. We also demonstrate a conserved mechanism of action of Nanos3, involving the interaction with the murine RNA-binding protein Pumilio2 and consisting of a potential translational repressor activity. According to the possible role of Nanos3 in inhibiting spermatogonia cell differentiation, we show that treatment with the differentiating factor all-trans retinoic acid induces a dramatic down-regulation of its expression. These results allow to conclude that, in the prepuberal testis, Nanos3 is important to maintain undifferentiated spermatogonia via the regulation of their cell cycle.

Keywords: Spermatogenesis; Spermatogonia; RNA-binding protein; Nanos; Pumilio

Introduction

Mammalian spermatogenesis is a complex process that includes cell proliferation, differentiation and morphogenesis. In the adult testis, spermatogonial stem cells, known as A single (A_s), represent a reservoir of germline stem cells (GSCs) that allows the spermatogenesis to continue throughout adult life. These are single cells localized on the basal membrane of the seminiferous epithelium of the adult testis and represent only 0.03% of total germ cells in the mouse testis (Tegelenbosch and de Rooij, 1993). The A_s stem cells proliferate and became A paired (A_{pr}), chains of two cells connected by intercellular bridges, and then A aligned (A_{al}), in which 4, 8, 16 cells and sometimes 32 cells are interconnected. A_s, A_{pr} and A_{al} are referred as undifferentiated spermatogonia. Approximately 6 days after birth, A_{al} cells begin to differentiate into A1 to A4, Intermediate and B spermatogonia, which finally undergo meiosis as primary spermatocytes (for a review see de Rooij, 2001). The appearance of A1 spermatogonia coincides with the expression of c-kit tyrosine-kinase receptor, a marker of the differentiating spermatogonia. The molecular mechanisms governing the decision of spermatogonial stem cells to proliferate, remaining undifferentiated cells or to differentiate into A1 cells, are largely unknown. Both intrinsic and extrinsic signals, coming from the surrounding Sertoli cells, affect this decision. For instance, growth factors produced by Sertoli cells are known to regulate spermatogonia proliferation/differentiation: the glial cell line-derived neurotrophic factor (GDNF) has

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been shown to be essential for spermatogonial stem cells self-renewing (Meng et al., 2000; Viglietto et al., 2000), BMP4 exerts both mitogenic and differentiative effects on undifferentiated spermatogonia (Pellegrini et al., 2003), while KL is required for proliferation and survival of differentiated c-kit-positive spermatogonia (Rossi et al., 1993; Dolci et al., 2001).

Although many efforts have been made to identify germ cell-specific genes that control spermatogonial stem cell proliferation/differentiation, up to now only few genes have been described. These genes, Plzf and Bcl6b, both encode for transcriptional repressors characterized by the BTB/POZ domain and are expressed in early undifferentiated spermatogonia. The transcriptional repressor Plzf has been shown to be required to regulate self-renewal and maintenance of the stem cell pool (Buaas et al., 2004) and its ablation, in mice, results in male infertility. Bcl6b has been demonstrated to be regulated by GDNF and to be crucial for spermatogonia stem cells self-renewal (Oatley et al., 2006). Other markers of undifferentiated spermatogonia such as Oct4, neurogenin3 and Sox3 have been identified, but a specific function has not been demonstrated (Ohbo et al., 2003; Raverot et al., 2005; Yoshida et al., 2004).

In lower organisms, the Nanos gene has been identified as important regulator of germline stem cell status. In *Drosophila* embryo, the Nanos gene (Nos) has been demonstrated to be required for proper development of primordial germ cells (PGCs). In the absence of maternal Nanos, PGCs fail to migrate into the gonad and do not become functional germ cells (Forbes and Lehmann, 1998; Kobayashi et al., 1996). In the *Drosophila* adult ovary, Nanos is expressed in the GSCs (Forbes and Lehmann, 1998) and regulates their self-renewal by inhibiting genes that promote GSCs differentiation into oocytes (Gilboa and Lehmann, 2004; Wang and Lin, 2004). Similarly, in zebrafish, Nanos orthologs regulate PGC survival (Koprunner et al., 2001) during early embryogenesis and are required to maintain oocyte production in adult ovaries (Draper et al., 2007).

Nanos encodes for a zinc-finger RNA-binding protein and shows a translational repression activity requiring the interaction with the ubiquitously expressed protein Pumilio. Pumilio is a member of the PUF family, an evolutionarily highly conserved family of RNA-binding proteins. The Nanos–Pumilio protein complex binds to Nanos-responsive element (NRE) in the 3′ UTR of target mRNAs and represses their translation (Asaoka-Taguchi et al., 1999; Sonoda and Wharton, 1999).

In the mouse, three homologs have been identified, Nanos1, 2 and 3, but only Nanos2 and Nanos3 are required for fertility. Nanos2 null-mice display a phenotype characterized by a complete absence of germ cells only in the testis. Nanos3 null-mice are characterized by a complete absence of germ cells in both sexes (Tsuda et al., 2003). In the developing gonad, Nanos3 is expressed in PGCs until 14.5 dpc in male gonad and until 13.5 dpc in female gonad; after this age its expression disappears and then it is found after birth only in male germ cells (Tsuda et al., 2003; Suzuki et al., 2007). Although knockout experiments strongly suggest an important role of this gene in mouse germ cell development, it remains undefined the role of Nanos3 in the postnatal testis.

In the present work, we have investigated the expression and the function of Nanos3 in mouse spermatogenesis. We show that, in prepuberal testis, Nanos3 is expressed specifically in undifferentiated spermatogonia and it is absent in differentiating c-kit expressing spermatogonia. We report evidences that Nanos3 plays a role in the maintenance of the undifferentiated state of germ cells regulating the spermatogonia cell cycle and inducing a prolonged transit in G1 phase. The molecular mechanism by which Nanos3 affects cell proliferation probably involves a repression of translation and the interaction with the RNA-binding protein Pumilio2.

Finally the observation that treatment of spermatogonia with the all-trans retinoic acid (ATRA), known to induce cell differentiation, causes a dramatic down-regulation of Nanos3 expression, is a further evidence for a role of Nanos3 in the maintenance of the undifferentiated state.

Materials and methods

Plasmid constructs

The complete ORF of mouse Nanos3 cDNA was amplified by RT-PCR using Proofstart polymerase (Pfu, Stratagene) and was cloned into pEGFP (pEGFP-C1; Clontech Laboratories) and pCDNA3N2-myc expression vectors. Pumilio2 complete ORF was cloned in pCMV-HA expression vector (Clontech Laboratories). The homology domain (HD) of Pumilio2 was cloned in pCDNA3N2-myc (myc-Pum2HD) and pGEX4T1 expression vector for GST-tagged Pum2HD (Clontech Laboratories). All cDNAs used in the experiments were sequenced by Cycle Sequencing (BMR, University of Padova, Italy).

Cell cultures and transfections

Spermatogonia were obtained from 4 and 7 dpp Swiss CD-1 mice, as previously reported by Rossi et al. (1993). Briefly, germ cell suspensions were obtained by sequential collagenase-hyaluronidase-trypsin digestions of freshly withdrawn testes. The suspension was preplated for 4 h in E-MEM with 10% FCS to facilitate adhesion of contaminating somatic cells to the plastic dishes. The resulting germ cell suspension was represented by 80% spermatogonia when using 7 dpp mice and by 50% spermatogonia when using 4 dpp mice. The cells were rinsed and further cultured for 24 h in E-MEM supplemented with 2 mM Na-pyruvate and 1 mM Na-lactate in the presence or absence of ATRA (0.3 μM; Sigma-Aldrich, Milan, Italy), GDNF, BMP4 and/or Kitl (100 ng/ml; Genzyme, MA). At the end of this culture period, most of the contaminating somatic cells attached to the dish, and floating spermatogonia were recovered in the suspension at a purity higher than 90%. Separation of c-kit-positive spermatogonia from c-kit negative spermatogonia was performed by using magnetic-activated cell sorting (MACS) with CD117 conjugated microbeads (Miltenyi Biotec, Germany). Isolated germ cells were then analyzed by immunofluorescence and semiquantitative RT-PCR.

Homogeneous populations (purity >90%) of spermatocytes and round spermatids were obtained from testes of 36 dpp mice by differential elutriation as previously described (Rossi et al., 2004). Sertoli cell monolayers from 7 to 17 dpp mice, devoid of contaminating germ cells, were prepared as previously described (Grimaldi et al., 1993).

Hek293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker Cambrex Bioscience), penicillin and streptomycin.

Transfection of Hek293 cells and spermatogonia was performed using lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. Briefly, 5×10^5 spermatogonia were resuspended in 100 μl of Opti-MEM® I Reduced-Serum Medium w/GlutaMAX™-I (Gibco-Invitrogen) and transfected with 0.25 μg of DNA of plasmids and 1 μl of Lipofectamine™2000 for 20 min. Cells were then washed 3 times in medium, and incubated in E-MEM with 10% FCS at 32 °C in 5% CO_2. Spermatogonia transfection efficiency was about 5%

and it was evaluated by FACS analysis after transfection with a plasmid containing the c-kit regulatory region driving the expression of EGFP (Cairns et al., 2003).

Northern blotting analysis

For Northern blot analysis, total RNA was extracted using the TRIzol reagent (Invitrogen), separated on a 1.5% agarose/formaldehyde gel and blotted onto nylon membrane (Hybond-N, Amersham, UK) in 10× saline sodium citrate (SSC) buffer. Nanos3, Pumilio2 and βactin probes were obtained by RT-PCR amplification. Hybridization was carried out following Quick Hybrid System's instruction (Stratagene, CA).

In situ hybridization

In situ hybridization experiments were carried out with digoxigenin-11-UTP-labeled probes (DIG RNA Labeling Mix, Roche Diagnostics), according to the instructions of the manufacturer. The antisense cRNAs, corresponding to the complete Nanos3 mRNA sequence until nt 710, was amplified by RT-PCR using oligonucleotides 5′TCTCTGCTCCTCTGCCCAGCCAT3′(forward) and 5′ TTGTGGTGTTGCCTCAACATCC 3′(reverse). The amplification product was cloned into pCR-Script vector (Stratagene) to obtain Nanos3 antisense and Nanos3 sense cRNAs. Briefly, samples of mouse testis at different ages were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h and washed in PBS 3 times for 10′. Samples were dehydrated, embedded in paraffin, sectioned at 6 μm and processed (Simeone et al., 1995).

Immunofluorescence, Western blotting, immunoprecipitation and GST Pull-down assay

In immunofluorescence assays, cell suspensions were left to adhere to poly-L-lysine-coated slides and permeabilized for 10 min in 0.1% Triton X-100 in PBS. After a 1 h block in 5% bovine serum albumin (BSA) in PBS, antibodies against Plzf (Chemicon, Milan, Italy), Oct4 (Santa Cruz Biotechnology, Inc.) or Sohlh1 (generously provided by Dr. A. Rajkovic) were added at a 1:100 dilution in 0.5% BSA in PBS and incubated overnight at 4 °C. Cyanin-3 secondary antibodies were added to the cells for 1 h and nuclei were labeled with Hoechst 33349 (1 μg/ml).

Cell protein extracts used for Western blot, immunoprecipitation or pull-down experiments, were obtained by resuspending cell pellets in lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris–HCl pH 7.5, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 0.5 mM NaVO4, protease inhibitor cocktail with 0.1% Triton-X-100), followed by centrifugation for 10 min at 12,000×*g* at 4 °C.

Immunoprecipitation from cell extracts of Hek293 cotransfected with pcDNA3-myc-Nanos3 and pCMV-HA-Pum2 (300 μg of total proteins) was performed after preclearing for 1 h with a mixture of Protein A- and Protein G-Sepharose beads (Sigma-Aldrich, Milan, Italy) in Interaction Buffer (IB) (20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 5 μM $ZnCl_2$, 0.5 mM DTT, 100 mM NaCl, 0.1% Tween 20, 1 mM dithiothreitol, protease inhibitor cocktail) to reduce the nonspecific binding. Supernatants, obtained by centrifugation for 3 min at 1000×*g*, were incubated with 1 μg of anti-HA antibody (Santa Cruz Biotechnology, Inc.) in IB for 2 h at 4 °C under constant shaking. Protein A-/Protein G-Sepharose beads, preadsorbed with 0.05% BSA, were incubated with the precleared cell extract for 3 h. Beads were then washed three times with the same buffer and absorbed proteins were eluted in SDS-sample buffer. Supernatants were loaded on 12% SDS-polyacrylamide gel electrophoresis as input.

Pull-down experiments were performed using GST fusion proteins purified from bacterial lysates on glutathione-agarose (Sigma-Aldrich), as previously described (Sette et al., 1998). Cell extracts (300 μg of total proteins) of transfected Hek293, precleared for 1 h with glutathione-agarose in IB, were added to 2 μg of GST protein or GST-Pum2HD fusion protein absorbed on glutathione-agarose in IB supplemented with 0.05% BSA. The incubation was performed in presence or absence of 1 μg of PBE-RNA (Pumilio Binding Element) (White et al., 2001) with 4 μl of RNase OUT inhibitor (40 U/ml, Invitrogen). After incubation for 90 min at 4 °C under constant shaking, beads were washed three times with the same buffer, and absorbed proteins were eluted in SDS sample buffer and resolved on a 12% SDS-PAGE for subsequent Western blot analysis. Supernatant was collected and loaded as input.

RNA pull down

To coimmunoprecipitate RNA bound to Nanos3–Pumilio2 complex, cell extracts (300 μg of total proteins) of Hek293 transfected with pCDNA3-myc-Nanos3, pCDNA3-myc-Pum2HD or pCDNA3-myc were precleared in IB for 1 h with a mixture of Protein A- and Protein G-Sepharose beads and immunoprecipitated as described above using anti-myc antibodies. Hence, beads bound to myc-Nanos3, myc-Pum2HD were washed three times and incubated with 7 dpp total testis RNA (10 μg for each sample) in the presence of 40 U/ml RNase OUT (Invitrogen) for additional 2 h at 4 °C. After three washes, bound RNA was eluted by extraction with phenol/chloroform and ethanol precipitated. Supernatant was collected and used as input. Immunoprecipitated RNA was labeled with γ^{32}P-ATP, using T4 Polynucleotide Kinase 10U/μl (Invitrogen) according to the manufacturer's instructions. Samples were separated on a nondenaturing 6% polyacrylamide gel in TBE buffer (Tris–Borate–EDTA). The gel was dried, and radioactivity was analyzed by autoradiography.

Polysome-RNPs fractionation by sucrose gradients

Isolated spermatogonia were transfected with appropriate plasmids (pCDNA3N2-myc-Nanos3, pCMV-HA-Pum2) and homogenized in lysis buffer supplemented with 40 U/ml RNase OUT. After 5 min of incubation on ice, the lysates were centrifuged for 10 min at 12,000×*g* at 4 °C. The supernatants were loaded on a 15–50% (wt/vol) sucrose gradients and sedimented by centrifugation for 110 min at 37,000 rpm in a Beckman SW41 rotor (Fullerton, CA). Each gradient was collected in 10 fractions and proteins were precipitated from each fraction as previously reported (Paronetto et al., 2006) and analyzed by Western blot.

FACS

Oct4-EGFP-positive spermatogonia were sorted from a germ cell suspension obtained from 2 dpp Oct4-EGFP mice resuspended in PBS, 2% BSA. We carried out cell sorting by using MoFlo sorter (DAKO, CA). Total mRNA was extracted and analyzed by RT-PCR.

Cell cycle was analyzed in spermatogonia after transfection with pEGFP-Nanos3 or with the empty vector as control. Cells were fixed in 1% paraformaldehyde for 30 min, washed in PBS and incubated for 16 h with 70% ethanol. After washing, cells were treated with RNAseA (10 μg/ml) for 30 min at 37 °C and then stained with propidium iodide (10 μg/ml) for additional 30 min at 37 °C in the dark. Stained cells were analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA).

Results

Nanos3 is expressed in undifferentiated spermatogonia and in ES cells

Although Nanos3 is required for mouse germ cells development (Tsuda et al., 2003), its role after birth is not known. In order to answer this question, we accurately studied the pattern of expression of Nanos3 during postnatal testis development.

Northern blot analysis (Fig. 1A) of RNA from total mouse testes from 1 dpp to adulthood showed that Nanos3 expression started early after birth, at 1 dpp, it increased until 7 dpp, and declined gradually afterwards, with the entrance of spermatogonia into meiosis and with the accumulation of spermatocytes and spermatids in the testis. However Nanos3 continues to be expressed at low level in the adult testis as observed by RT-PCR

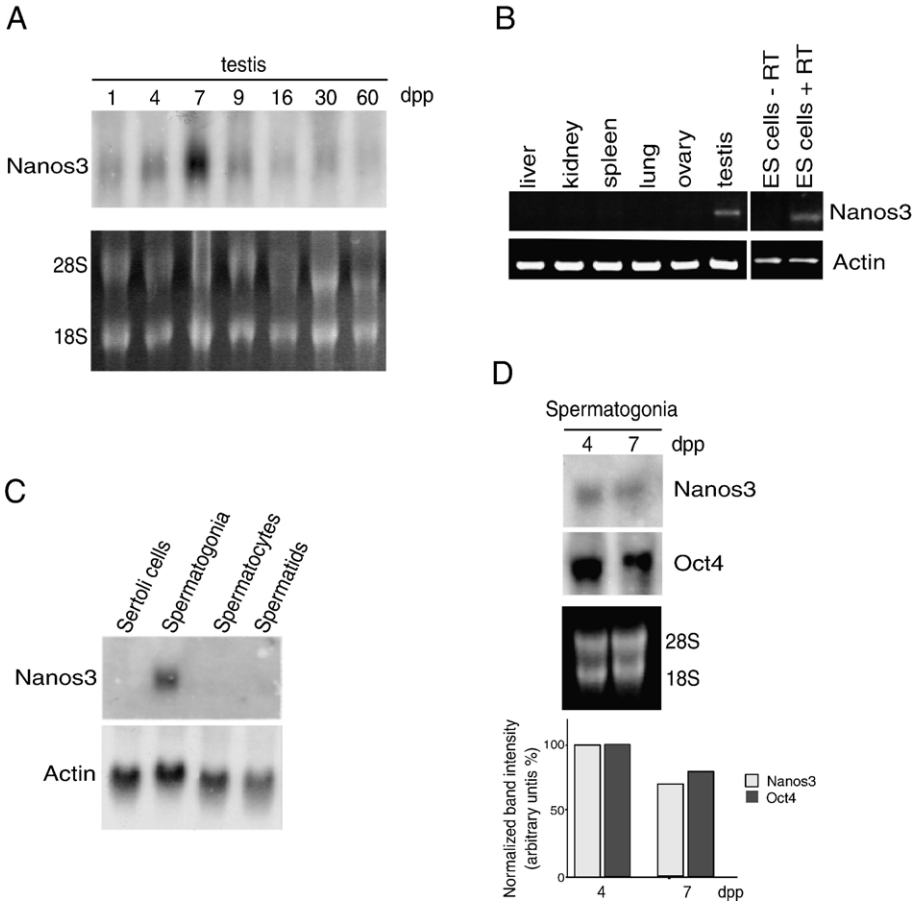


Fig. 1. Nanos3 expression in developing testis. (A) Expression of Nanos3 mRNA in developing testis revealed by Northern blot analysis. Total RNA, 20 μg, extracted from mouse testis at different ages were separated on 1.5% agarose gels, transferred onto nylon membranes and probed with labeled Nanos3 cDNA. (B) Nanos3 expression in mouse adult tissues and ES D3 cell line by RT-PCR. (C) Nanos3 expression within isolated germ cell populations at different stage of differentiation, by Northern blot. (D) Northern blot analysis of Nanos3 and Oct-4 mRNAs in spermatogonia from 4 dpp and 7 dpp mouse testes. Differences in mRNA expression have been evaluated by densitometric analysis and normalized for 28-18S RNA. In panel D, the histogram showing differences of Nanos3 and Oct4 expression in 4 dpp and 7 dpp spermatogonia (arbitrary units) are reported. Actin mRNA or 28S-18S is shown as loading reference.

(Fig. 1B). Interestingly, while other tissues tested are completely negative, the embryonic stem cell line D3 expressed Nanos3, indicating that this protein may represent a marker of undifferentiated state (Fig. 1B).

To better characterize the expression pattern of Nanos3 in germ cells, we performed Northern blot analysis on purified testicular cells at different stages of differentiation. As shown in Fig. 1C, Nanos3 was detected only in 7 dpp spermatogonia, while pachytene spermatocytes, spermatids and Sertoli cells were completely negative. Since the population of spermatogonia obtained from 7 dpp mice is heterogeneous and includes mitotic germ cells at various stages of differentiation, from A_s to A1–4, Int and B cells, we compared Nanos3 expression of spermatogonia from testes at 4 dpp, when the undifferentiated spermatogonia are the prevalent germ cell population, with that from 7 dpp testes, when differentiating c-kit-positive cells are also present (Dolci et al., 2001). By densitometric analysis, we found that Nanos3 mRNA showed a 1.4-fold increase in spermatogonia from 4 dpp mice with respect to that detected in spermatogonia from 7 dpp mice (Fig. 1D), suggesting that its expression correlates with the undifferentiated stage. To support this evidence is the observation that Oct4, a marker of undifferentiated spermatogonia, showed a similar expression pattern in spermatogonia at 4 and 7 dpp.

In order to localize in the testis the cell types expressing Nanos3 mRNA, we performed in situ hybridization experiments on testicular sections at 1 dpp, 7 dpp and adult testes using digoxigenin-labeled probes. At birth, spermatogonia, which are defined gonocytes, occupy a central position within the tubules and move toward the periphery when spermatogenesis starts. At around 3 dpp, they are found in the periphery of the seminiferous tubules, resume mitosis and give rise to the undifferentiated spermatogonia (Nagano et al., 2000). At around 5 dpp, the first differentiated c-kit-positive cells are found within the tubules (Manova et al., 1990). Fig. 2 shows that, at 1 dpp, Nanos3-positive spermatogonia are located in the center of the seminiferous tubules, while, at 7 dpp, we found only few Nanos3-expressing spermatogonia located at the periphery of the tubules that likely represent the subset of undifferentiated spermatogonia.

In contrast to RT-PCR data, in the adult testis we were not able to identify any Nanos3-expressing cells.

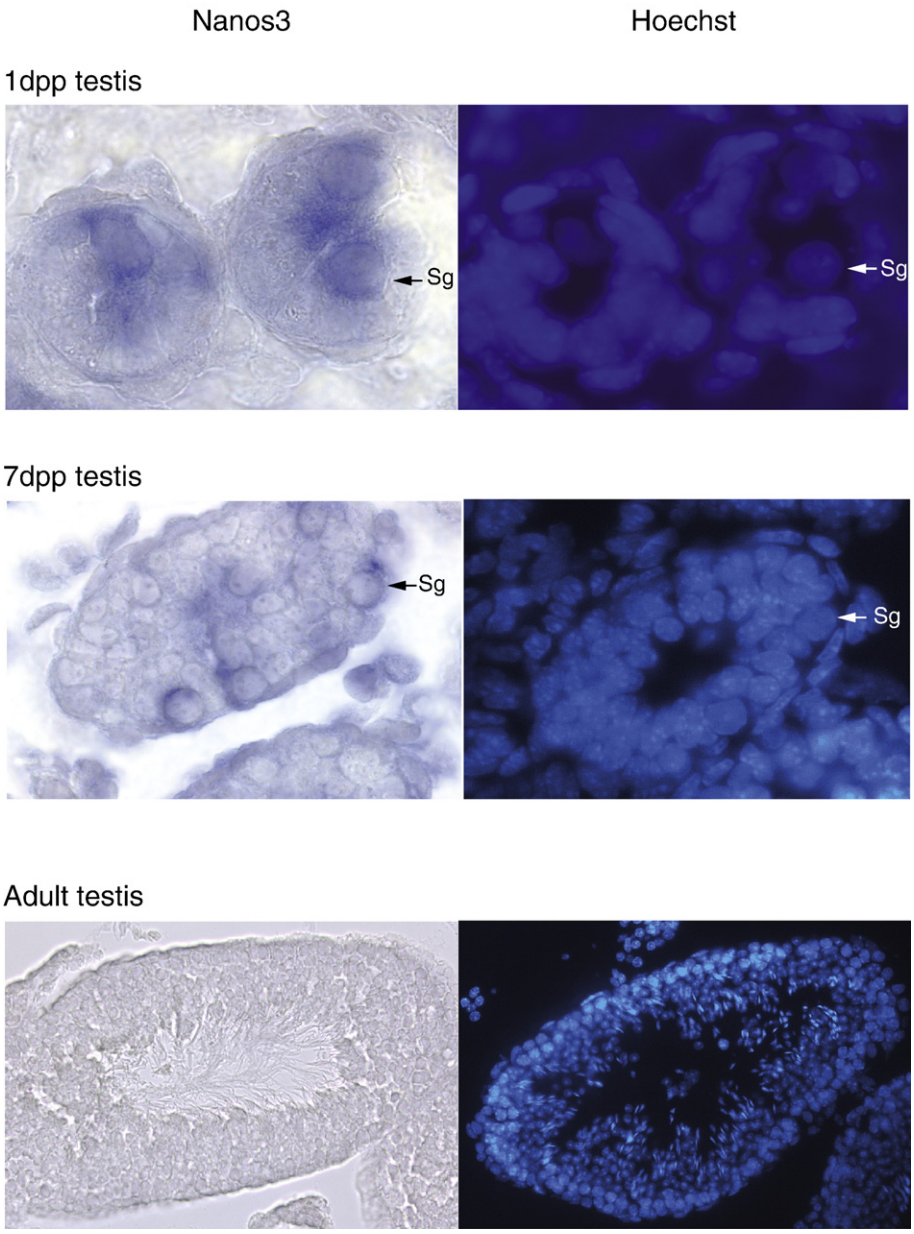
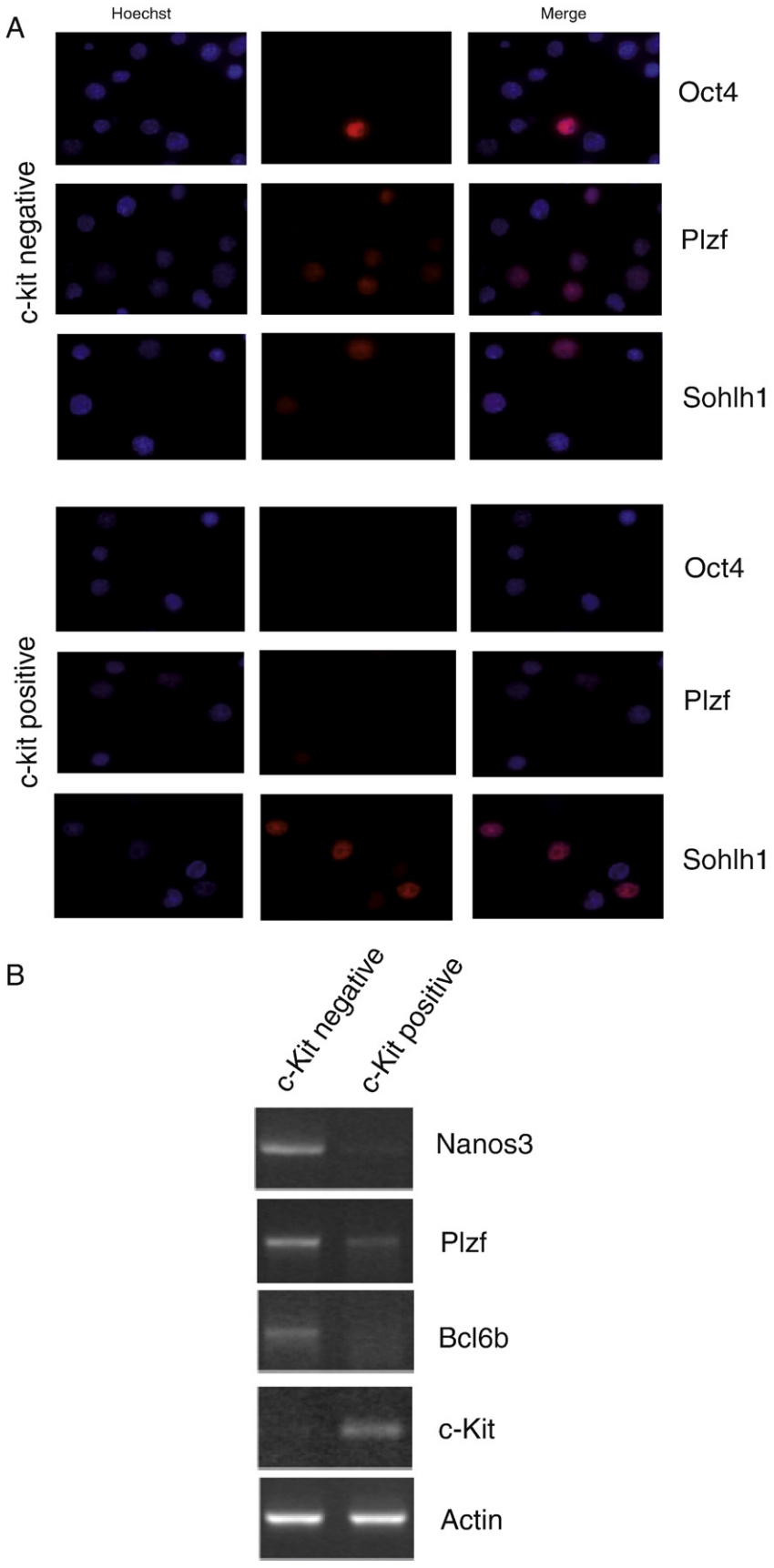


Fig. 2. Testicular expression of Nanos3. *In situ* hybridization of 1 dpp, 7 dpp and adult testis sections, using as probe a digoxigenin-11-UTP-labeled antisense Nanos3 corresponding to the sequence from nt 1 to nt 710. Sections of 1 dpp, 7 dpp and adult testes stained with Hoechst 3332 to detect nuclear morphology are shown. Arrows indicate the flattened nuclear morphology of a Nanos3-positive spermatogonia in the middle of the seminiferous tubule at 1 dpp and spermatogonia at the basement membrane at 7 dpp (Sg).

To demonstrate that Nanos3 is expressed in this subset of spermatogonia, we isolated c-kit-positive spermatogonia and c-kit-negative spermatogonia from 6 dpp testes, by using magnetic-activated cell sorting (MACS) with CD117-conjugated beads. Immunofluorescence characterization of the two populations showed a correct pattern of staining with Oct4 and Plzf, markers of undifferentiated c-kit negative spermatogonia and with Sohlh1 (spermatogenesis and oogenesis basic helix–loop–helix transcription factor 1), whose expression is most significant in differentiating c-kit-positive spermatogonia (Fig. 3A) (Ballow et al., 2006). Semiquantitative RT-PCR analysis of mRNA extracted from the two cell populations showed that, as expected, Nanos3 expression was detected in the c-kit negative population together with other markers of undifferentiated spermatogonia like Plzf, Oct4 and Bcl6b mRNAs (Fig. 3B), clearly indicating that Nanos3 expression is confined to undifferentiated cells and it is absent in differentiating c-kit-positive cells.

To further characterize the Nanos3-expressing cells, we chose to investigate the coexpression with Oct4 which is expressed in early undifferentiated spermatogonia at prepubertal stages. Spermatogonia derived from 2 dpp Oct4-GFP transgenic mouse testes (Hübner et al., 2003) were sorted on the basis of specific GFP expression (Fig. 4A). The sorted GFP^{+}


A
Hoechst
Merge
c-kit negative
Oct4
Plzf
Sohlh1
c-kit positive
Oct4
Plzf
Sohlh1
B
c-Kit negative
c-Kit positive
Nanos3
Plzf
Bcl6b
c-Kit
Actin

cell population expressed Oct4 as shown in Fig. 4B, suggesting that the GFP expression paralleled the endogenous expression of Oct4. RT-PCR analysis of the mRNA extracted from the GFP^+ spermatogonia showed that these cells coexpressed Nanos3 with Plzf, Bcl6b and Oct4 (Fig. 4C). The GFP^- fraction contained mainly testicular somatic cells (data not shown).

The interaction between Nanos3 and Pumilio2 is conserved

In *Drosophila*, the molecular mechanism by which Nanos regulates translation involves its interaction with the protein Pumilio, a member of a highly conserved family of RNA-binding proteins (Puf family) (Asaoka-Taguchi et al., 1999). The Nanos–Pumilio complex recognizes and binds to specific nucleotide sequence in the 3-UTR of target mRNAs acting as translational repressor during development and differentiation. In mouse, two Puf proteins, *Pumilio1* and *Pumilio2*, have been identified. *Pumilio1* and *Pumilio2* share 51 and 55% overall similarity with the fly *Pumilio*, whereas their RNA-binding regions, the homology domain (HD), show a very high degree of evolutionary conservation (86 and 88% respectively). Both genes are expressed in a variety of tissues suggesting that they have widespread function. In the testis, Pumilio2 is mainly expressed as two transcripts, a larger 6 kb and a smaller 4 kb transcript, which probably arise through alternative splicing, but it is not known which cell type in the testis express the gene (White et al., 2001).

We report in Fig. 5A that Pumilio2 is ubiquitously expressed in the testis: spermatogonia express mainly the 6Kb transcript, spermatocytes express both the 6 kb and 4 kb transcripts, while spermatids express only the smaller 4 kb transcript. Moreover, the 6 kb Pumilio2 specific band is found also in Sertoli cells.

To investigate the possibility that in mouse Nanos3 could interact with Pumilio2 *in vivo*, we performed immunoprecipitation experiments. Hek293 were cotransfected with both myc-Nanos3 and HA-Pum2, encoding the complete ORF of Pumilio2, and cell extracts were immunoprecipitated using either nonimmune rabbit IgGs or anti-HA IgGs. As shown in Fig. 5B, anti-HA IgGs immunoprecipitated a significative amount of myc-Nanos3. Nonspecific IgGs did not precipitate Nanos3, indicating that the interaction between Pumilio2 and Nanos3 is specific. In *Drosophila*, the formation of Pumilio–Nanos complex is mediated by the presence of RNA (Murata and Wharton, 1995). By contrast, we found that in mammals the treatment of cell extracts with RNAse did not affect the interaction between the two proteins indicating that the formation of the complex is RNA-independent.

Furthermore in order to test whether the murine protein Nanos3 or its homolog Nanos2 could physically interact with Pumilio2 *in vitro*, we used purified recombinant proteins in pull-down assays. GST or GST-Pum2HD, which contains the homology RNA-binding domain of the protein and has been demonstrated to be sufficient for protein interaction (Jaruzelska et al., 2003), was expressed in *E. coli*, preadsorbed to glutathione-agarose beads and then incubated for 90′ with cell extracts of Hek293 transfected with myc-Nanos3, myc-Nanos2 and myc-Pum2HD. After several washes, the adsorbed proteins were eluted and analyzed in Western blot by using the anti-myc antibody. As shown in Fig. 5C, GST-Pum2HD bound specifically to all the proteins, whereas no binding to GST alone was observed. Coomassie blue staining of the gel demonstrated that the amount of GST proteins used for the pull down was comparable (data not shown). Moreover the physical interaction between Nanos3 and Nanos2 with Pum2HD is not affected by the presence of RNA sequences containing the murine Pumilio Binding Element (PBE), while the addition of PBE decreases the interaction of Pumilio2 with itself. These results confirm that Nanos3 and Pumilio2 can form a protein–protein interaction *in vitro* even in the absence of RNA.

The RNA-binding protein Nanos3 is found in ribonucleoparticles

In *Drosophila*, the Nanos–Pumilio complex recognizes and binds to specific sequences in 3′-UTR regions of mRNAs target. To investigate the RNA-binding properties of the murine Nanos3-Pumilio2HD complex, we performed RNA pull down experiments. Hek293, that express endogenous Pumilio2 but not Nanos3 (data not shown), were transfected with myc-Nanos3 or with myc-Pum2HD and immunoprecipitation was performed using anti-myc IgGs. The immunoprecipitated recombinant proteins, which eventually might coimmunoprecipitate endogenous proteins and/or RNA, were then incubated for 90′ with total RNA from 7 dpp testes. After several washes, bound RNA was extracted using phenol-chloroform, ethanol precipitated, labeled by γ^{32}P-ATP and separated on nondenaturing polyacrylamide gel. As shown in Fig. 6, immunoprecipitated myc-Nanos3 (lanes 3 and 4) bound detectable amounts of RNA only when RNA from 7 dpp total testis was added to the reaction (lane 3). On the contrary, immunoprecipitated myc-Pum2HD (lane 2) did not bind neither endogenous RNA nor RNA from testis. The same result was obtained using HA-Pum2 that encode the complete Pumilio2 ORF (data not shown). These results are indicative of the capacity of Nanos3, alone or together with endogenous Pumilio2, to bind specific RNA of germ cells. In control cells (mock), that have been transfected only with the empty vector, no specific band was detected (lane 5).

In order to investigate whether the RNA-binding protein Nanos3 could be a translational repressor in spermatogonia, we examined its subcellular distribution and its association with RNPs. Cell extracts from spermatogonia transfected with myc-

Fig. 3. Nanos3 is expressed in undifferentiated c-kit negative spermatogonia. (A) Immunofluorescence localization of Oct4, Plzf and Sohlh1 in differentiating c-kit-positive and undifferentiated c-kit negative spermatogonia. Spermatogonia c-kit-positive and c-kit-negative were separated from a total spermatogonia pool, obtained 6 dpp mouse testes, by using CD117 conjugated immunomagnetic beads. (B) Total RNA from c-kit-positive and from c-kit negative spermatogonia was extracted and analyzed by semiquantitative RT-PCR for the expression of the indicated genes.

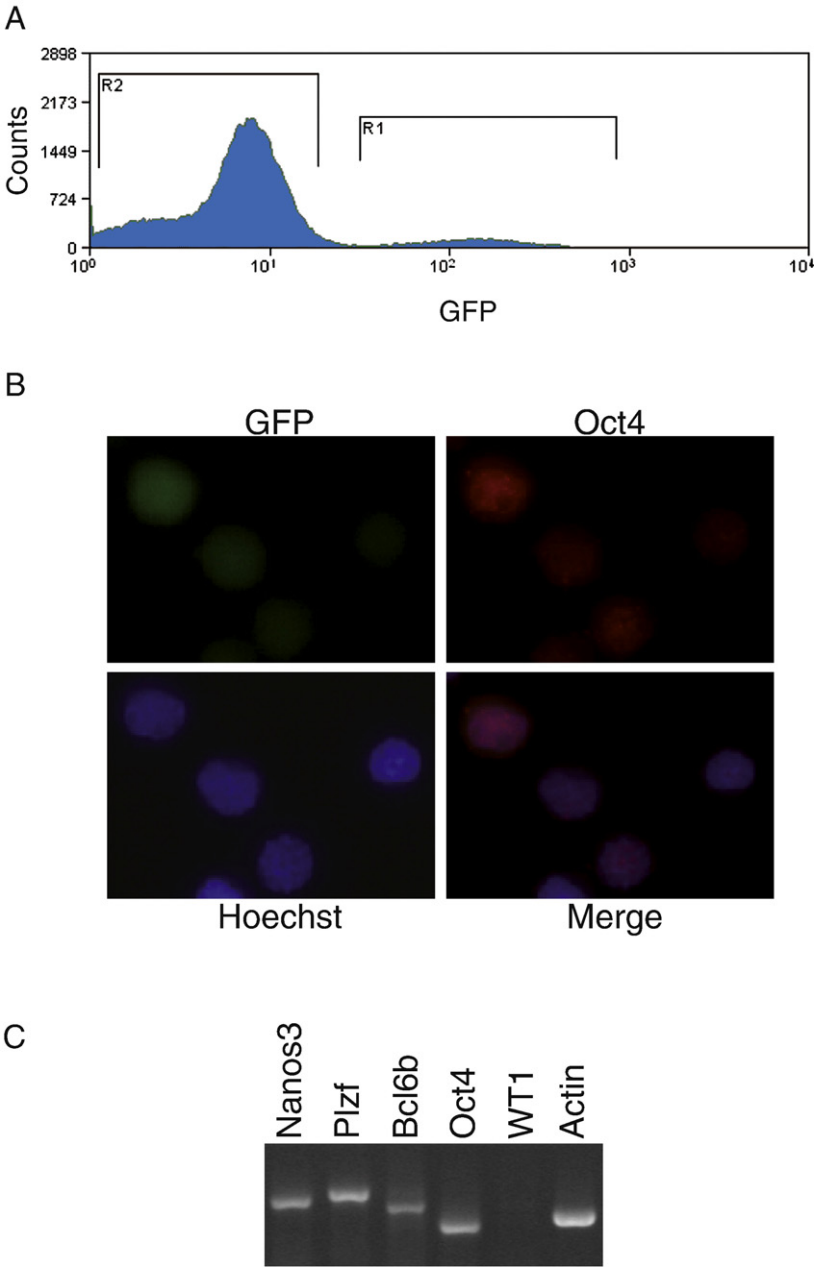


Fig. 4. Nanos3 is expressed in Oct4-positive spermatogonia. (A) Oct4-GFP spermatogonia were isolated from a germ cell suspension from 2 dpp Oct4-GFP mice by flow sorting. Graphic represents the profile of the GFP-positive sorted population (R1). (B) Immunofluorescence localization of endogenous Oct4 on sorted Oct4-GFP spermatogonia. (C) Total RNA from the sorted Oct4-GFP spermatogonia was analyzed by RT-PCR to assess the expression of the indicated genes.

Nanos3 alone or together with myc-Pum2 HD, were fractionated on 15–50% sucrose density gradient that allows to separate the polysome fraction from the RNPs fraction. Samples were recovered and analyzed by Western blotting with anti-myc, anti-HA and anti-S6 antibodies.

Fig. 7 shows that Nanos3 was found in fractions of lower density where RNPs are localized while it is completely absent from the fractions containing polysomes or ribosomal subunits. As expected, transfected Pumilio2, revealed by anti-myc antibody, and endogenous Pumilio2, revealed by anti-Pumilio2 antibody, are found associated to RNPs in the same fractions where Nanos3 is present. These data suggest that Nanos3 associate with RNPs together with Pumilio2, behaving like an RNA-binding protein with translational repression activity.

Nanos3 over-expression inhibits cell proliferation

The biological role of Nanos3 in spermatogonia is completely unknown. In *Drosophila*, it has been demonstrated that Nanos–Pumilio complex affects cell cycle progression, by inhibiting the transition from G2 phase to mitosis in the migrating pole cells during development (Forbes and Lehmann, 1998). We hypothesized a possible function of Nanos3 in cell cycle regulation of undifferentiated spermatogonia. To

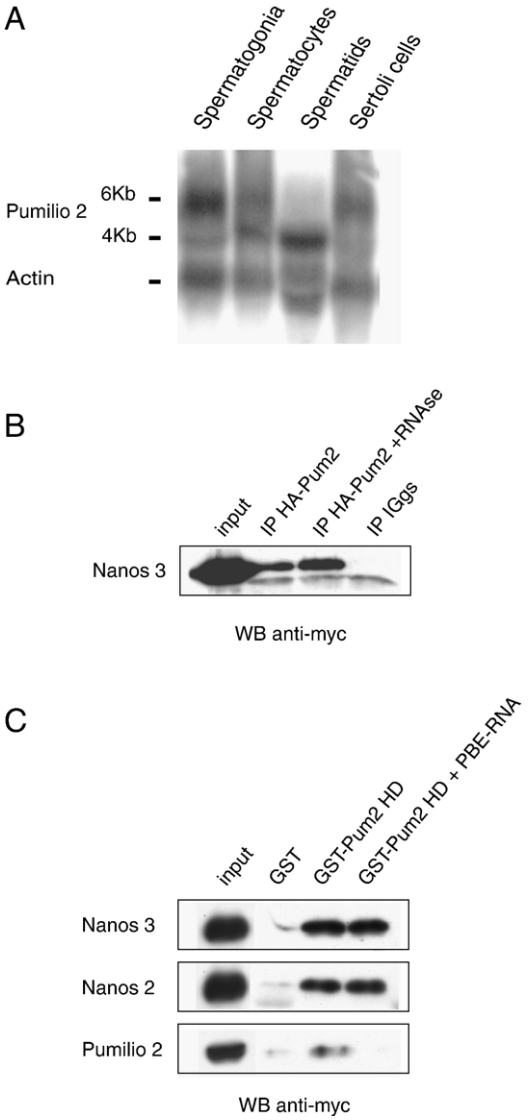


Fig. 5. Nanos3 associates *in vitro* and *in vivo* with Pumilio2. (A) Northern blot analysis of Pumilio2 mRNA in testicular germ cells. The two transcripts at 4 kb and 6 kb are indicated. (B) Cell extracts of Hek293 cotransfected with pCDNA3-myc-Nanos3 and pCMV-HA-Pum2 were used in immunoprecipitation experiments using anti-HA antibody or IGgs. Coimmunoprecipitated Nanos3 protein was detected by Western blot using mouse anti-c-*myc* antibody. Sample of cell extract treated with RNase is indicated. (C) Pull-down experiments were performed using purified GST and GST-Pum2HD fusion protein expressed in *E. coli*, adsorbed on glutathione-agarose beads and incubated with extracts of Hek293 cells transfected pCDNA3-myc-Nanos3, pCDNA3-myc-Pum2HD or pCDNA3-myc-Nanos2. Bound proteins were revealed by Western blot analysis using anti-myc antibody. In the indicated sample, 1 μg PBE-RNA (Pumilio Binding Element) (White et al., 2001) was added.

verify this possibility, highly purified spermatogonia from 7 dpp mice were transfected with pEGFP or pNanos3-EGFP for 24 h and then analyzed by FACS after propidium iodide staining. We found that pEGFP transfected spermatogonia as well as nontransfected control cells were prevalently in G1 phase (70% of the cells) and in a smaller percentage in the S/G2/M phase (27%). Interestingly, overexpression of Nanos3 increased the G1 phase spermatogonia population (75% of the cells) and decreased the S/G2/M phase fractions (21%) (Fig. 8B). The percentage of G1 phase cells was significantly ($P=0.028$) increased in Nanos3-EGFP transfected cells compared with cells transfected with only EGFP. These data indicate that overexpression of Nanos3 affects spermatogonia cell cycle, by inducing a prolonged transit in G1 phase and probably maintaining a mitotic quiescence in undifferentiated spermatogonia.

Retinoic acid regulates Nanos3 expression

Proliferation and differentiation of spermatogonia are regulated by different growth factors and cytokines. Among these factors, ATRA, the active metabolite of vitamin A, is known as a potent mitogen and differentiating factor. More recently, different observations suggest a role of ATRA in

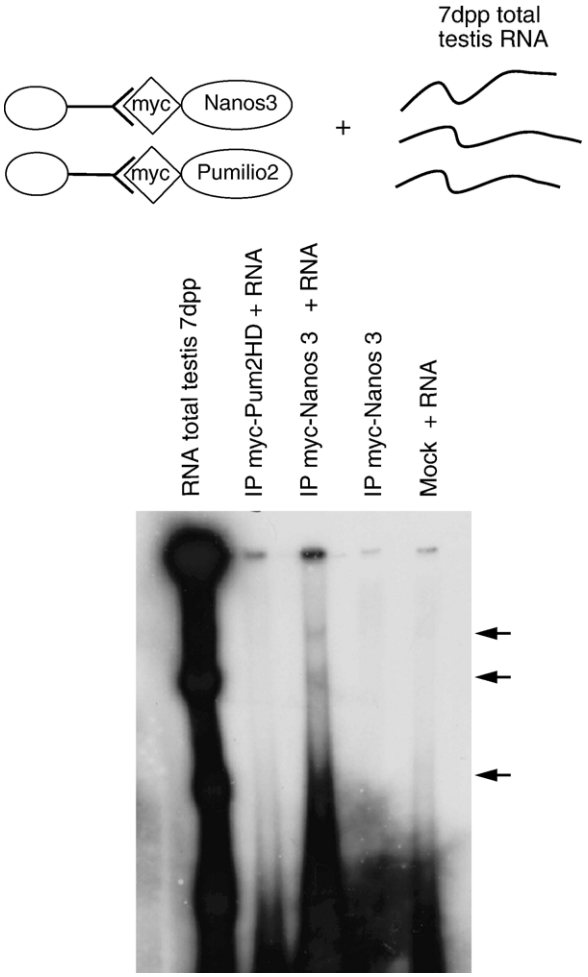


Fig. 6. RNA pull down assay. Cell extracts of Hek293 transfected with pCDNA3-myc-Nanos3 (lanes 3–4), pCDNA3-myc-Pum2 HD (lane 2) or pCDNA3-myc (lane 5) were immunoprecipitated with anti-c-*myc* antibody and incubated with 7 dpp total testis RNA (10 μg). Coimmunoprecipitated RNAs were detected by labeling with T4 Polynucleotide kinase. Samples were separated on a nondenaturing 6% polyacrylamide gel in TBE buffer and radioactivity was analyzed by autoradiography. In lane 1, the pattern of labeled total testis RNA is shown.

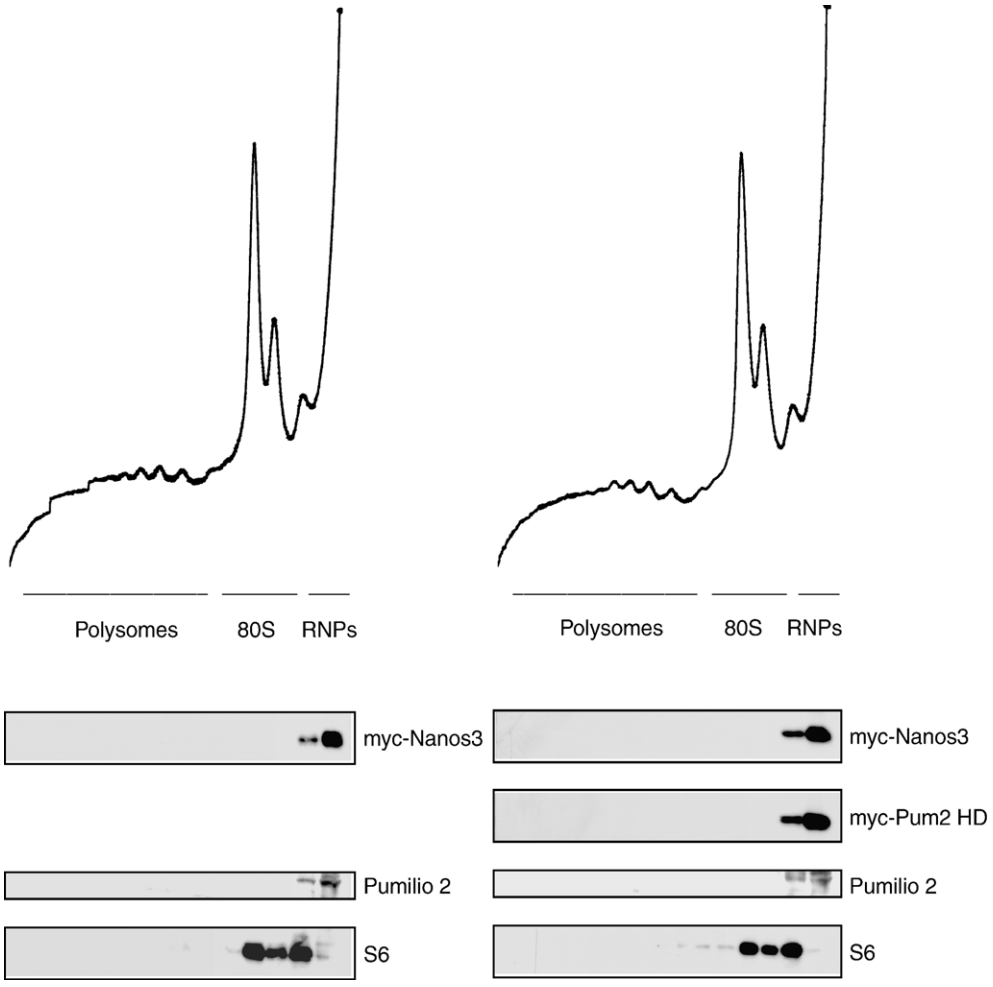


Fig. 7. Nanos3 associates with RNPs in mouse spermatogonia. Fractionation on sucrose gradient of cell extracts from spermatogonia transfected with pCDNA3-myc-Nanos3 alone (left panel) or together with pCDNA3-myc-Pum2 HD (right panel). Absorbance profiles at 254 nm show the distribution of soluble RNPs, single ribosome and polysomes. The distribution of Nanos3, Pumilio2 and of the ribosomal S6 protein, used as standard of distribution of the ribosome (80S), was analyzed by Western blot analysis of each fraction, using anti-myc and anti-S6 antibodies. Anti-Pumilio2 antibodies (Abcam, Cambridge, UK) were used to identify the endogenous protein.

triggering the progression of undifferentiated spermatogonia toward a more differentiated cell type (de Rooij, 2001; Livera et al., 2002; Wang and Culty, 2007).

Since our data indicated that Nanos3 has a role in regulating the undifferentiated state of spermatogonia, we were interested to investigate whether ATRA regulated Nanos3 expression in these cells. Isolated spermatogonia were cultured in the presence of 0.3 μM ATRA for 2 h and 24 h and Nanos3 expression was analyzed by RT-PCR and Northern blotting. Interestingly, after 2 h of treatment, we found a light decrease in Nanos3 expression that became dramatically down-regulated after 24 h (Figs. 9A, B). We chose, as control, the expression of Stra8, a cytosolic protein present in premeiotic testicular germ cells known to be induced by ATRA (Oulad-Abdelghani et al., 1996) (Fig. 9C). On the other hand, we observed that the expression of Pumilio2 is not regulated by ATRA in spermatogonia (Fig. 9D).

Other growth factors that have been reported to affect spermatogonia proliferation, such as BMP4 and GDNF, did not regulate Nanos3 expression (Fig. 9A).

Discussion

In this work we have investigated the expression of Nanos3 in developing postnatal mouse testis and its role in spermatogenesis. Maintenance of spermatogenesis depends on the presence in the testis of germ stem cells capable of self renewing or able to undergo differentiation. However, the molecular mechanisms governing the cellular process defining the stem cell fate are poorly understood.

In this paper, we present data suggesting that the translational repressor Nanos3 is a regulator of the undifferentiated state of spermatogonia. Although the role of Nanos3 in germ cell development in the embryo has been clearly shown by knockout mice, this is the first evidence of a role of Nanos3 in mouse spermatogenesis after birth.

We first show that Nanos3 is expressed specifically in the postnatal testis in mitotic germ cells. By Northern blot analysis, Nanos3 expression is detectable in total testis, as early as 1 dpp, it increases until 7 dpp and then gradually decreases with the progression of spermatogenesis. The in situ hybridization

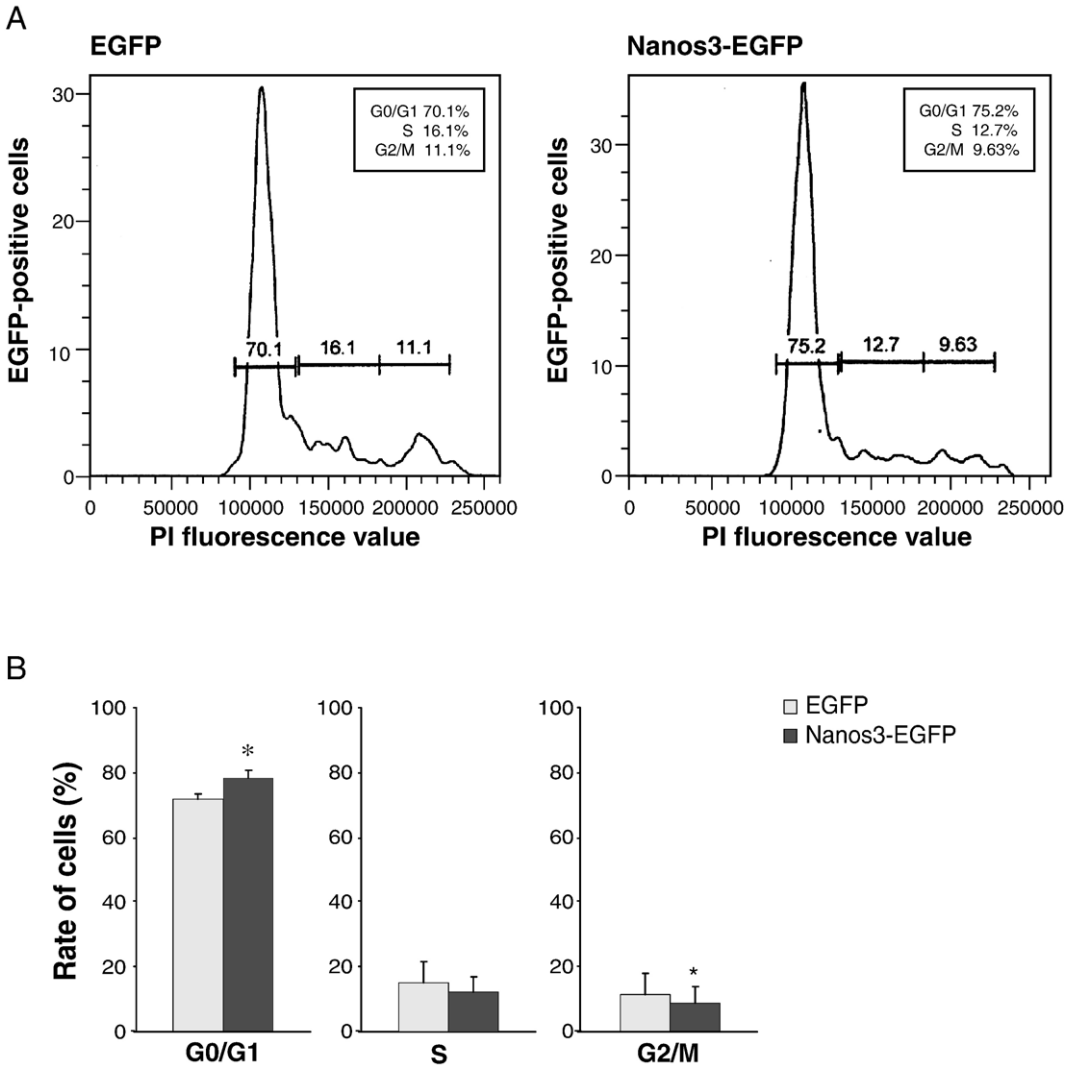


Fig. 8. Nanos3 overexpression results in an increase in G1 phase spermatogonia. (A) 7 dpp spermatogonia transfected with pNanos3-EGFP or pEGFP-C1 were fixed, stained with propidium iodide and analyzed for DNA content on a FACS Calibur Flow Cytometer. A representative experiment is shown. (B) Histograms show the percentage of spermatogonia in the G0/G1 phase [73.6±3.2%, mean±SEM; $n=7$ (pNanos3-EGFP cells) vs. 69.2±2.3%; $n=7$ (pEGFP cells)], S phase and G2/M phase [16.4±4.1%, mean±SEM; $n=7$ (pNanos3-EGFP cells) vs. 11.1±2.4%; $n=7$ (pEGFP cells)]. Asterisks denote significant differences ($P\leq 0.05$).

experiments showed that Nanos3-expressing germ cells are located in the center of the seminiferous tubule at 1 dpp, they are found at the periphery of the tubules, in a small population of spermatogonia characterized by large nuclei with disperse chromatin, at 7 dpp, while positive cells were not found in adult testes. However, since we found a faint signal by RT-PCR, we cannot rule out that in the adult testis Nanos3-expressing cells could represent a very rare subpopulation of undifferentiated spermatogonia and thus not easily detectable by in testis sections. Alternatively it is possible that Nanos3 mRNA is expressed at very low level in undifferentiated spermatogonia of the adult testis, below the sensitivity threshold of the in situ hybridization.

Since we observed that Nanos3 mRNA decreases in isolated spermatogonia from 4 dpp to 7 dpp, we tried to identify the Nanos3-expressing cells within the whole mitotic germ cell population. In fact in prepuberal testis, spermatogonia are a heterogeneous population of undifferentiated, differentiating and premeiotic germ cells. It is known that at 4–5 dpp undifferentiated spermatogonia are the major component of germ cells, and that from this age, differentiating c-kit-expressing spermatogonia start to appear. We separated c-kit-negative from c-kit-positive spermatogonia from 6 dpp testes, by using c-kit-conjugated immunomagnetic beads and we confirmed the purity of these cell populations, by detecting the expression of known specific markers such as Oct4 and Plzf for undifferentiated spermatogonia, and Sohlh1 mainly for differentiating cells. According to our previous observations, we found that Nanos3 mRNA is expressed in the undifferentiated kit-negative spermatogonia.

It has been previously shown that, after birth, Oct4 is expressed only in spermatogonial stem cells (Pesce et al., 1998). To better understand if, within the undifferentiated spermatogonia population, the Oct4-positive cells were also expressing Nanos3, we sorted GFP-positive spermatogonia from Oct4-GFP mouse testes. Interestingly we found that Oct4-positive

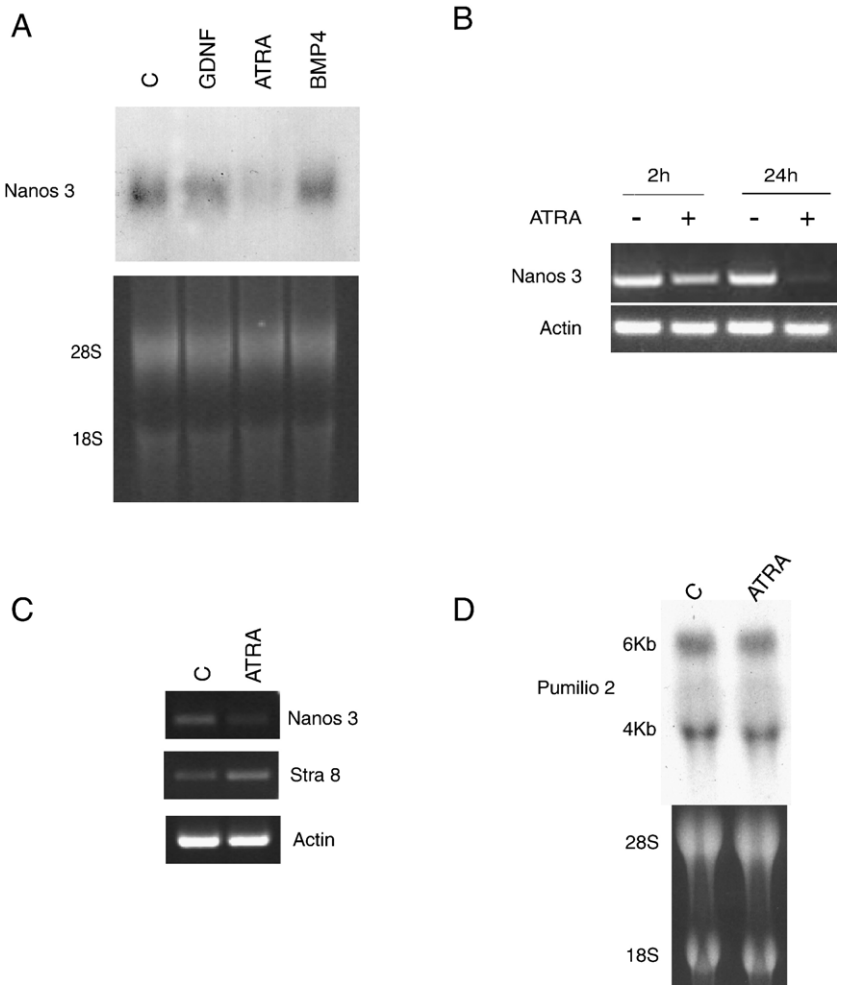


Fig. 9. Regulation of Nanos 3 expression. (A) Northern blot analysis of Nanos3 mRNA from 7 dpp spermatogonia untreated or treated with ATRA, GDNF and BMP4 for 24 h. (B) RT-PCR analysis of Nanos3 mRNA from 7 dpp mouse spermatogonia treated with ATRA for 2 or 24 h. (C) RT-PCR analysis of Nanos3 and Stra8 mRNA from 7 dpp spermatogonia treated with ATRA for 24 h. (D) Northern blot analysis of Pumilio2 in 7 dpp spermatogonia treated with ATRA for 24 h. Actin was used as control loading.

spermatogonia also expressed Nanos3 mRNA together with Plzf and Bcl6b.

Taken together, these data demonstrate that, in prepuberal mouse testis, Nanos3 marks undifferentiated spermatogonia starting from the A_s cells but it is excluded from differentiating c-kit-positive cells.

Tsuda et al. suggested that during gonadal development and high levels of expression of Nanos3 are correlated to a proliferating state of germ cells. Indeed, a lack of Nanos3 expression is found when germ cells stop to divide as in fetal gonocytes (14.5 dpc) or begin to differentiate as in fetal ovocytes (13.5 dpc).

The presence of Nanos3 in undifferentiated spermatogonia might have to do with a possible role in the control of the cell cycle and in the maintenance of the stem cell phenotype. In adult ovary of *Drosophila* and zebrafish, Nanos orthologs are important for self-renewal of GSCs by inhibiting genes that promote GSCs differentiation into oocytes (Gilboa and Lehmann, 2004; Wang and Lin, 2004; Draper et al., 2007).

One possible mechanism by which Nanos3 could maintain the undifferentiated state of spermatogonia is a regulation of their cell cycle. Indeed it has been reported that, in *Drosophila*, Nanos is involved in inhibiting the transition from G2 phase to mitosis in GSC (Forbes and Lehmann, 1998). Furthermore, it has been demonstrated (Groisman et al., 2002; Miskimins et al., 2001; Kim et al., 2003) that the RNA-binding proteins play an important role in the regulation of the cell cycle. The G1 phase represents a critical step of the cell cycle during which the decision to proliferate, to differentiate or to revert to a quiescence state, is taken (Pardee, 1989). It has been proposed that a prolonged transit in G1 phase is an adult stem cell property and that a slower progression of the cell cycle could be required for the self-renewal and maintenance of stem cells (Nygren et al., 2006). Interestingly we find that over-expression of Nanos3 in spermatogonia induces a modest but significant increase (over 5–7%) in the G0/G1 population consistent for a potential role of this RNA-binding protein in maintaining the stem cell character. A slower progression through the G1 phase seems not to be limited to spermatogonial stem cells. It has, in fact, been shown also in adult mouse hemopoietic stem cells (HSCs) (Nygren et al., 2006).

In summary we demonstrate that Nanos3 is expressed in undifferentiated spermatogonia and influences the pattern of their cell cycle. The observation that over-expression of Nanos3 induces a lower proliferative activity should be consistent with the requirement to maintain the stem cell population and to avoid their exhaustion. This function is likely achieved by Nanos3 by repressing the translation of mRNAs involved in the control of the cell cycle, whose identity is up now completely unknown. Interestingly in *Drosophila* germ plasm Nanos is involved in the translational repression of cyclin B (Asaoka-Taguchi et al., 1999).

In addition, we report evidences for a conserved molecular mechanism of action of Nanos3 in mouse spermatogenesis. We demonstrate that Nanos3 interacts *in vitro* and *in vivo* with Pumilio2, an RNA-binding protein ubiquitously expressed in the testis, also when the homology Puf domain of Pumilio2 is used. The interaction between Nanos and Pumilio has been already characterized in others organisms: in *Drosophila* embryo (Sonoda and Wharton, 1999), in *C. elegans* germ line (Zhang et al., 1997; Kraemer et al., 1999), in *Xenopus* oocytes (Nakahata et al., 2001) and finally Jaruzelska et al. (2003) also reported the interaction between human NANOS1 and human PUMILIO-2.

Moreover we show for the first time that Nanos3 is actually able to bind mRNA from germ cells. Although the identity of the mRNA targets as well as the actual biological roles of Nanos3 is completely unknown, we find that Nanos3 is mainly detected in the nontranslating (storage) messenger ribonucleoproteins (mRNP) fraction, in mouse spermatogonia. We expect that proteins found in mRNPs are involved in the translational repression of specific mRNAs. Hence our results are consistent with a role of Nanos3 in maintaining the stem cell stage by repressing translation of specific mRNAs that are probably required for cell cycle progression and differentiation.

Treatment of spermatogonia with the differentiative factor ATRA has allow to gather further evidences for the role of Nanos3 in the maintenance of the undifferentiated state of the cells in which it is expressed. The effects of ATRA, the active metabolite of vitamin A, on spermatogenesis has been clearly demonstrated by vitamin A-deficient mice that are affected by testicular degeneration with a complete disappearance of all meiotic and postmeiotic germ cells (van Pelt and de Rooij, 1990) and with an arrest at the stage of A spermatogonia (Huang and Hembree, 1979). Recent data showed that, in the developing ovary, retinoic acid signaling is responsible for the induction of germ cell meiosis and it has been suggested that the testis uses the same signaling system of the ovary to induce germ cell meiosis, but at different times (Bowles et al., 2006). However, previous data (de Rooij, 2001; Livera et al., 2002; Wang and Culty, 2007; Ghyselinck et al., 2006) and our unpublished results (Filipponi et al. manuscript in preparation) indicate that, in male gonad, retinoic acid (ATRA) plays a critical role in controlling the spermatogonia differentiation from A_{al} to A1.

Our observation that ATRA treatment induces a strong decrease in Nanos3 expression, indicates that high levels of Nanos3 are associated with the undifferentiated state. Furthermore since A_s cells have not been demonstrated to be a target of ATRA, our data suggest that this regulation possibly involves the A_{al} spermatogonia.

Taken together, our results indicate, for the first time, a possible role for Nanos3 in the postnatal testis in maintaining the undifferentiated state of spermatogonia by using a molecular mechanism of action conserved from flies to male mice. Whether this role is essential for the maintenance of the stem cells reservoir in the adult testis should be elucidated by conditional gene ablation in gonocytes.

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